AN 1954:78020 CAPLUS
DN 48:78020
OREF 48:13774e-g
TI The histochemical basis of metachromasia
AU Walton, K. W.; Ricketts, C. R.
CS Univ. Birmingham, UK
SO Brit. J. Exptl. Pathol. (1954), 35, 227-40
DT Journal
LA Unavailable
AB **Dextran sulfates**, **carboxymethyl** dextrans, **carboxymethyl dextran sulfates**, 3 prepns. of heparin, chondroitin sulfate, hyaluronic acid, and the sulfates of chitin, xylan, alginic acid, and pectin were used with toluidine blue to investigate metachromasia. Quant. detn. shows that the intensity of the metachromasia varies with the no. and dissocn. of the acid groups but not the no. or the nature of the constituents of the polymer. It is probably dependent on the soly. characteristics of the dye-substrate complex. Changes in metachromasia in tissues indicate changes in the no. of available acid groups but not in the degree of polymerization of the polysaccharide.

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INVESTIGATION OF THE HISTOCHEMICAL BASIS OF METACHROMASIA.

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Received for publication February 5, 1954.

WHEN histological sections are stained with certain basic dyes, some tissue components assume a colour which differs from that of the dye. This phenomenon was called "metachromasia" by Ehrlich. Subsequently, this property has been found to be particularly characteristic of mucopolysaccharides.

The intensity of metachromasia may vary in certain circumstances. For example it has been reported that: (i) the metachromatic staining of the intercellular material normally demonstrable in granulation tissue is reduced or absent in scorbutic animals (Penney and Balfour, 1949) and can be abolished by treatment of normal granulation tissue with the hyaluronidases (Campani and Reggiani, 1950); (ii) the intensity of metachromasia of the ground substance of connective tissues varies with altered functional states (Gersh, 1951); (iii) mucins elaborated by various mucus-secreting tumours differ in the intensity of their metachromasia (Lennox, Pearse and Richards, 1952); and (iv) the metachromasia of the ground substance of cartilage varies in intensity with ageing (Loewi, 1953).

It has been suggested repeatedly that this variation in the intensity of metachromasia may connote depolymerization of the mucopolysaccharide components of the tissues (Gersh, 1951; Lennox *et al.*, 1952; Loewi, 1953).

Quantitative studies on the interaction between the basic thiazine dye toluidine blue and a number of synthetic and naturally-occurring acidic polysaccharides were undertaken to investigate the physico-chemical mechanism of metachromasia and, in particular, to investigate the above-mentioned hypothesis.

MATERIALS.

Derivatives of dextran.

Dextran is a complex glucose polymer consisting, in the natural state, of many hundreds of glucose units. Partial acid hydrolysis of native dextran and fractionation with acetone yield a series of dextrans differing in mean molecular weight (and therefore in extent of polymerization) as judged by differences in intrinsic viscosity. From dextrans derived in this way three series of derivatives were prepared:

(a) *Dextran sulphates.*—Esterification was effected by treatment of the dextran with chlorosulphonic acid in pyridine followed by isolation of the dextran sulphate as the sodium salt in each case. The chemical, physical and biological characteristics of these compounds have been described previously (Ricketts, 1952*a*; Walton, 1952, 1953).

(b) *Carboxymethyl dextrans.*—The dextran fractions were treated with monochloracetate and sodium hydroxide and the carboxymethyl dextrans isolated as the sodium salts. The number of carboxyl groups per glucose unit in the products was estimated approximately from the percentage of copper in a copper salt formed from each compound.

(c) *Carboxymethyl dextran sulphates.*—Sodium carboxymethyl dextran was converted to a pyridine salt by passage through a cation exchange resin and neutralization with pyridine. The pyridine salt was isolated as a dry powder and sulphated in the same way as the dextran.

Heparins.

The following preparations were employed: (a) International Standard heparin, kindly supplied by Dr. W. L. M. Perry, Director of the Bureau of Biological Standards, National Institute for Medical Research, London; (b) "Pularin" (Evans) batch no. N10090, and (c) Heparin (Lederle) batch no. NP115–128. The two last compounds were in the form of their sodium salts.

Chondroitin sulphate.

A sample of purified material was kindly supplied by Dr. W. J. C. Dyke, Evans Biological Institute, Runcorn, Cheshire.

Hyaluronic acid.

A purified sample was kindly provided by Dr. R. J. Boscott, Department of Anatomy University of Birmingham.

Miscellaneous synthetic polysaccharide sulphuric esters.

The following preparations were obtained through the courtesy of the individuals named: Xylan sulphate ("Thrombocid")—Dr. R. Marx, Medical Clinic of the University of Munich. Xylan sulphate ("T.S.144")—Professor E. J. Wayne, Department of Pharmacology, Sheffield University. Polymannuronic acid (alginic acid) sulphate ("Paritol")—Mr. T. B. Wallace, Smith, Kline and French, Inc. Polygalacturonic acid (pectin) sulphate ("Treburon")—Dr. L. Berger, Roche Products, Ltd. Chitin sulphate—Dr. R. H. Barnes, Sharp and Dohme, Inc. Some of the chemical and physical characteristics of the above compounds are shown in Table I.

TABLE I.—*Sources and Properties of Reagents Used.*

Compound.	Biological source.	Per cent N.	Per cent S.	Specific optical rotation.
International standard heparin	Ox tissue	4·33	12·45	+60°
Heparin (Evans batch no. N10090)	Bovine lung	1·96	8·61	+42·3°
" (Lederle batch no. NP115–128)	" "	1·95	9·60	+33·9°
Chondroitin sulphate	Bovine cartilage	5·0	2·96	−32·7°
Hyaluronic acid	Human umbilical cord	4·34	<0·05	−26·7°*
Chitin sulphate	Crab shells	2·81	12·0	−23·7°
Xylan sulphate ("Thrombocid")	Wood	—	15·6	−55°
" " (B.D.H. "T.S.144")	"	—	15·85	−96·5°
Alginic acid sulphate ("Paritol")	Seaweed	—	11·2	−64·7°
Pectin sulphate ("Treburon")	Soft fruit	—	13·37	+128·8°

* Determination made on sample after removal of small amount of insoluble material.

Toluidine blue.

It has been shown by Ball and Jackson (1953) that commercial samples of this dye vary in composition and in their capacity to give satisfactory metachromasia in histological use. Toluidine blue (Colour Index no. 925), batch no. 008480, supplied by Hopkin and Williams, Ltd., London, was found to be satisfactory for histological purposes. The nitrogen content of this sample, determined by the micro-Kjeldahl method, was 8·41 per cent, equivalent to 61 per cent toluidine blue. Chromatographic separation by the method of Ball and Jackson showed that the metachromatic activity resided in three fractions which could be differentiated easily from contaminants which showed little or no affinity for the acidic polysaccharides examined.

The original (impure) dye was used, except where otherwise specified, as a 0·005 per cent (w/v) solution in 0·01 N HCl containing 0·2 per cent (w/v) sodium chloride.

Spectrophotometric measurements were made in the Unicam Spectrophotometer Model S.P.500 (Cambridge Instrument Co.) using 1 cm. glass cells.

Absorptiometric measurements were made in the Spekker Photo-electric Absorptiometer, Type H760 (Hilger and Watts, Ltd.) using 1 cm. glass cells and Ilford 607 (orange) filters.

RESULTS.

Effect of Solutions of Acidic Polysaccharides on Toluidine Blue.

On adding increasing concentrations of a naturally-occurring sulphated polysaccharide, such as heparin, to a constant dilute concentration (0·005 per cent) of toluidine blue, the colour of the dye changes from blue through purple to reddish-violet. Examination of the mixtures in a spectrophotometer shows accompanying alterations of the absorption-curve (Fig. 1). It will be seen that

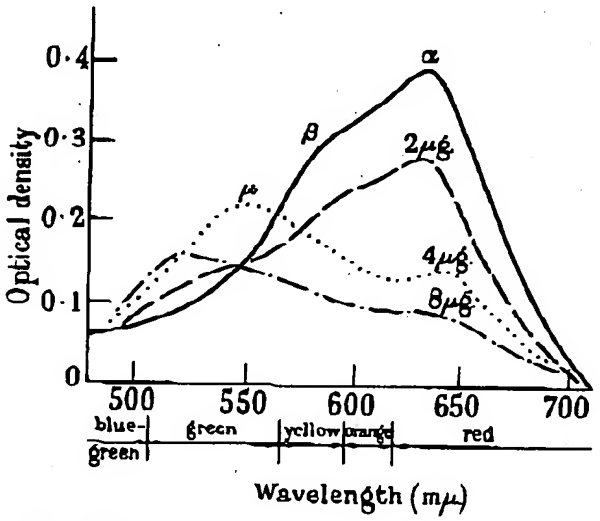

FIG. 1.—Alterations in the absorption curve of toluidine blue (final concentration 0·0025 per cent in 0·2 per cent sodium chloride at pH 2·04) on the addition of varying concentrations of heparin.

——— = toluidine blue solution alone.
– – – – = toluidine blue solution + 2 μg. heparin.
. . . . . = toluidine blue solution + 4 μg. heparin.
– . – . – = toluidine blue solution + 8 μg. heparin.

the dye solution alone shows maximal absorption of the longer wave-lengths (of orange and red light) and transmission of the shorter wave-lengths, hence appearing blue. The peak of the absorption curve occurs at 630 mμ. On adding heparin the absorption curve alters with increase of heparin concentration in that successively more intense absorption of yellow and then of green light occurs. The eye therefore sees the complementary colours, purple, and then red. New absorption maxima occur at about 590 mμ and between 560 and 540 mμ.

Similar changes were found to occur with other acidic polysaccharides (dextran sulphate, chondroitin sulphate) but differences were noted in the heights of the peaks at 630 mμ (α band), 590 mμ (β band) and between 560 and 540 mμ (μ band) with equal weights (2–8 μg.) of these compounds.

With high concentrations of strongly-charged acidic polysaccharides the metachromatic complex precipitates from solution. If allowed to stand, the particles of the precipitate sediment and the supernatant solution can be seen to be markedly decolourized. The altered solubility of the substrate-dye complex can also be demonstrated at low concentrations of added acidic polysaccharide by shaking the mixture with petroleum ether. The metachromatic or bound component separates as a layer at the interface leaving the unbound dye in solution. Spectrophotometric examination of the aqueous phase then shows an absorption curve similar to that of the dye alone, but the peak in the α-band is of diminished amplitude as compared with the peak of the original dye solution. The difference between the optical density of the treated solution and that of the original is thus a measure of the amount of dye bound in the metachromatic complex.

This is the basis of MacIntosh's (1941) method in which a series of mixtures of varying concentrations of acidic polysaccharide with a constant concentration of dye is treated in this way. When this method was used and the concentration of substrate was plotted against the percentage of the dye bound, different slopes

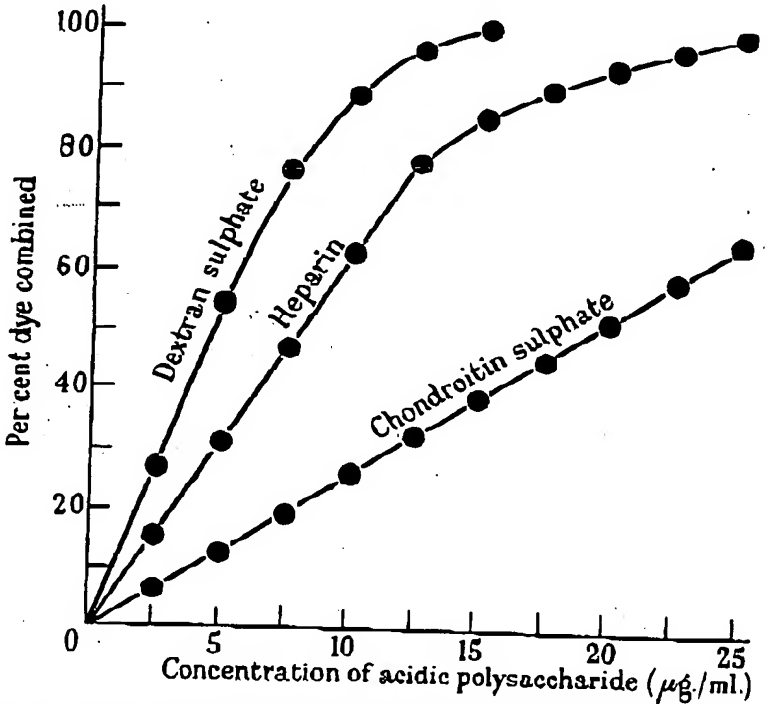

FIG. 2.—Relation between the concentration of acidic polysaccharide added and the amount of dye bound and removed from solution by petroleum ether extraction.

were obtained for different acidic polysaccharides (Fig. 2). To facilitate quantitative comparison of the metachromatic activity of these compounds, a preparation of heparin was adopted as an arbitrary standard. From the data plotted as in Fig. 2, the concentration (s) of the standard heparin relative to that (t) of any other acidic polysaccharide giving the same colour change could be obtained. Comparison of a series of ratios (s/t) obtained from varying percentages of dye

bound by any given acidic polysaccharide showed that, in most cases, the ratios were constant within 2–5 per cent over the linear portions of the curves. Where greater variation occurred, the highest value of s/t was taken. The heparin standard was assigned an arbitrary value of 100 colour units. The metachromatic activity of other compounds was then expressed in these units by simple derivation from the value of the ratio s/t (*e.g.*, if the ratio s/t for heparin and compound X was $\frac{1}{2}$, the metachromatic activity of compound X was expressed as 50 colour units).

Comparison of Metachromatic Activity of Acidic Polysaccharides.

Using the method outlined above, experiments were designed to investigate the molecular characteristics influencing metachromatic activity. Derivatives of dextran were employed for the initial experiments since these compounds were characterized as regards their degree of polymerization and the nature and number of their acidic groups.

Experiment 1.—A dextran fraction containing about twenty glucose units was sulphated to varying degrees. The metachromatic activity of the resulting series of compounds was estimated. If metachromatic activity depended solely upon the degree of polymerization of a given sulphated polysaccharide, these compounds might have been expected to exhibit equal activity since they all originated from the same parent dextran. However, it became evident that they did not show equal activity. On relating their metachromatic activity, on the other hand, to the extent of their sulphation, a linear relation was found (Fig. 3).

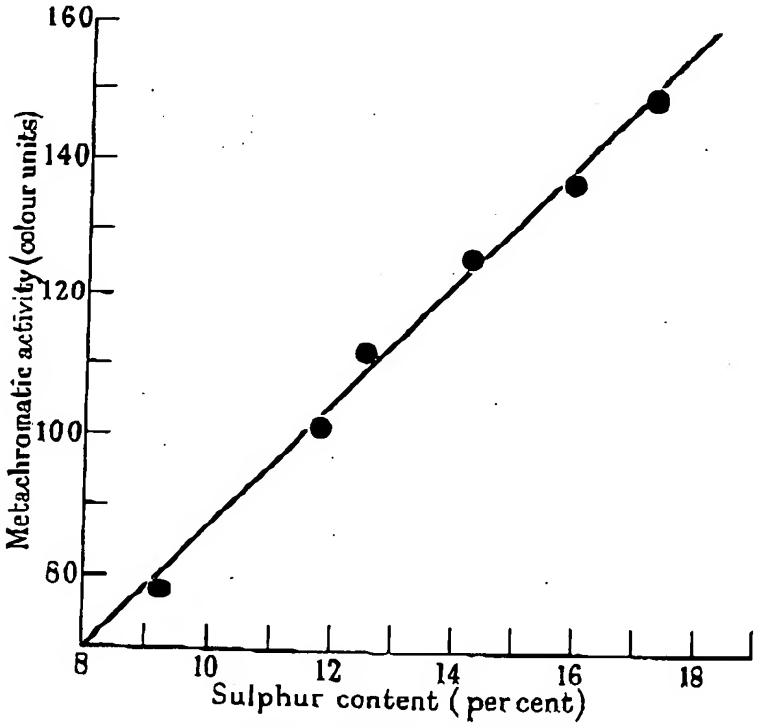

FIG. 3.—Relation between metachromatic activity and sulphur content of a series of dextran sulphates prepared from the same parent dextran (containing about 20 glucose units) but varying in their degree of sulphation.

Experiment 2.—The compounds used in Exp. 1 were paired with other dextran sulphates of approximately equal sulphur content but of widely different degrees of polymerization. The metachromatic activity of these pairs was compared (Fig. 4). It will be seen that the compounds with equal sulphur content showed equal metachromatic activity regardless of the divergence in molecular size between the components of each pair.

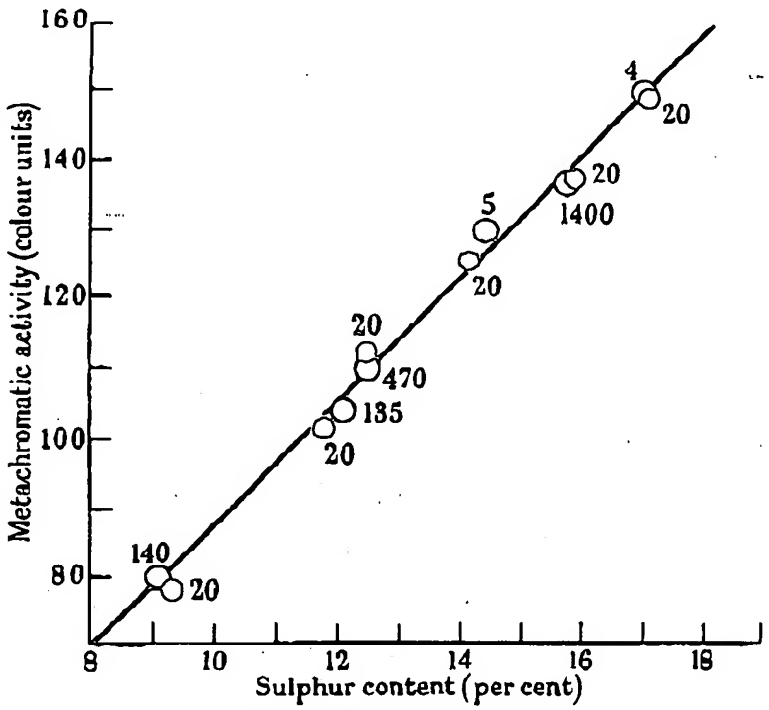

FIG. 4.—Relation between metachromatic activity and sulphur content of pairs of dextran sulphates of approximately equal sulphur content but of differing degrees of polymerization.
O = Dextran sulphates containing approximately 20 glucose units.
O = Dextran sulphates of varying molecular size. The number beside each point indicates the approximate number of glucose units in the compound.

Experiment 3.—This experiment was undertaken to see whether the relation between sulphur content and metachromatic activity found in the previous experiments held good over an even wider range of variation of sulphur content and molecular size. A range of glucose polymers was investigated which varied in sulphur content between 2·15 and 20 per cent and in size from glucose sulphate to a dextran sulphate containing approximately 6000 glucose units. Once more, a linear relation was obtained between sulphur content and metachromatic activity for compounds containing between 6000 and 4 glucose units (see Fig. 5). But sulphate esters of the trisaccharide (malto-triose). the disaccharide (maltose) and monosaccharide (glucose) showed progressively further departure from the curve obtained for the higher polymers.

The whole range of compounds showed some heparin-like anticoagulant activity *in vitro*. But compounds containing < 5 glucose units showed only transitory anticoagulant activity *in vivo*, presumably because they were almost immediately

excreted in the urine (Ricketts, 1952*b*). It may be assumed that such compounds would diffuse equally readily through other capillary membranes and would therefore not be demonstrable in the tissues by ordinary histological methods. From this it follows that the metachromatic activity of larger non-diffusible compounds which would be demonstrable histologically would not indicate their degree of polymerization.

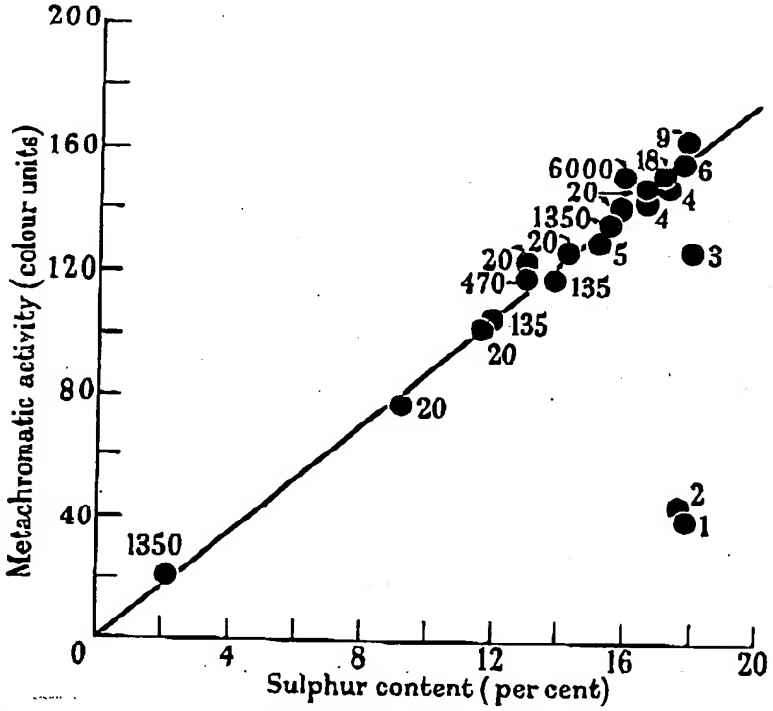

FIG. 5.—Relation between metachromatic activity and sulphur content of a range of glucose polymers of varying molecular size. The number beside each point indicates the approximate number of glucose units in the compound.

Influence of the Nature of the Acidic Radicle.

Sulphate groups are not the only acid radicles responsible for metachromasia. Other workers have shown that polyphosphates (Wiame, 1947; Weissman, Carnes, Rubin and Fisher, 1952) and silicates (Kelley and Miller, 1935; Merrill, Spencer and Getty, 1948; Curran, 1953) are also effective. Wislocki, Bunting and Dempsey (1947) and Meyer (1947) reported that hyaluronic acid (in which carboxyl groups were the only acidic radicles present) was metachromatic in high concentrations. Another experiment was undertaken to compare the activity of polycarboxylates and polysulphates.

Experiment 4.—In this experiment, the metachromatic activity of three glycollic acid ethers of dextran (carboxymethyl dextrans) and of a sample of purified hyaluronic acid, was compared with that of the standard heparin. The toluidine blue solution was used, as before, at pH 2·04, and also in barbiturate buffer at pH 7·3 (Michaelis, 1930). It can be seen from Table II that, at pH 2·04, the carboxylated dextrans showed some activity but that to obtain a μ-

absorption band, concentrations several hundred times greater than those of the corresponding sulphate esters were necessary.

TABLE II.—*Metachromatic Activity of Carboxylated Polysaccharides Compared with that of Heparin.*

Compound.	Average number of glucose units.	COOH per glucose unit.	Colour value. At pH 2·04.	Colour value. At pH 7·3.
Carboxymethyl dextran A	1350	0·79	0·10	0·24
" " B	100	1·0	0·32	0·68
" " C	20	1·1	0·33	0·74
Hyaluronic acid	?	?	0·58	1·10
Standard heparin	—	—	100·00	100·00

The sample of hyaluronic acid showed slightly greater metachromatic activity than the carboxylated dextrans. This sample of hyaluronic acid was found to be sulphur-free by the method of analysis used, but Sylvén and Malmgren (1952) using a method of analysis for sulphate of great sensitivity demonstrated that traces of sulphate-containing polysaccharides (?chondroitin sulphates) are common contaminants of hyaluronate preparations. It is possible that slight contamination of this sort might have accounted for the present findings. Sylvén and Malmgren also showed that with increased purification of hyaluronate and decrease of sulphur content, there was a direct relation with decrease in the intensity of metachromasia.

At pH 7·3, the metachromatic activity of the polycarboxylates was approximately double that at pH 2·04 but was still very considerably less than that of the polysulphates. Once again it appeared that the number of acidic groups rather than the extent of polymerization was the factor determining the intensity of metachromasia.

Investigation of Miscellaneous Polysaccharide Sulphuric Esters.

The naturally-occurring mucopolysaccharides are not simple hexose or pentose polymers but conjugates containing uronic acids and amino-sugars. In order to investigate whether the structure of the parent polysaccharide itself influenced metachromatic activity and to see whether the results obtained with the dextran sulphates were applicable to the sulphuric esters of more complex mucopolysaccharides, the following sulphated compounds were investigated: (*a*) Pentosans—two preparations of xylan sulphate; (*b*) Naturally-occurring polyuronic acids—polygalacturonic acid sulphate and polymannuronic acid sulphate; (*c*) Synthetic polycarboxylates—sulphate esters of the carboxymethyl dextrans used in Exp. 4; (*d*) Naturally-occurring conjugates of hexuronic acid and hexosamine—two samples of heparin and a sample of chondroitin sulphate; (*e*) A naturally-occurring polymer of glucosamine—chitin sulphate.

Experiment 5.—The sulphur contents of these compounds and their metachromatic activities were estimated by the methods previously employed. The result of plotting metachromatic activity against sulphur content was compared with the result obtained in Exp. 3 for the dextran sulphates (Fig. 6). The points representing the activity of these very diverse compounds did not fall exactly upon the curve obtained for the dextran sulphates but it seemed clear that a similar relation held good.

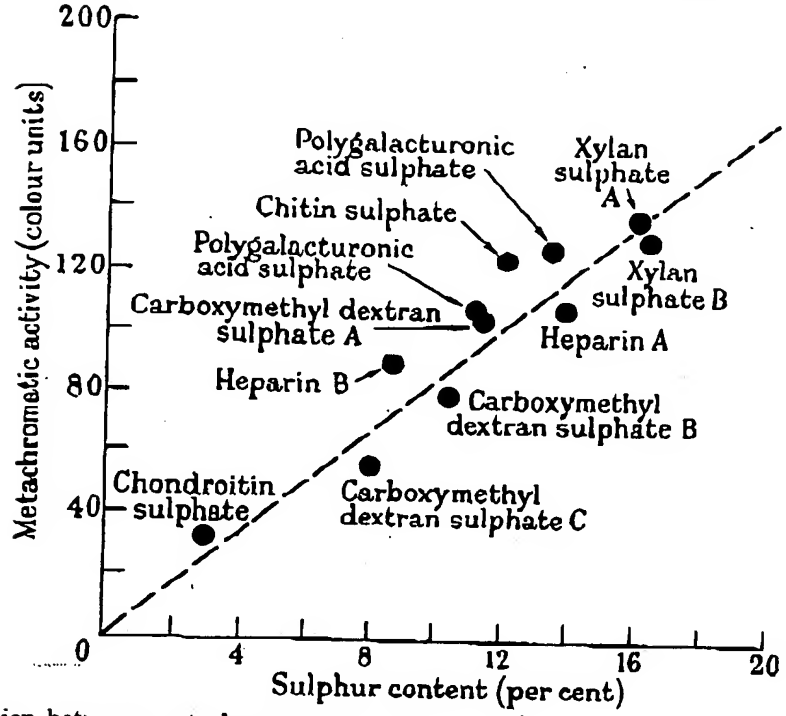

FIG. 6.—Relation between metachromatic activity and sulphur content of various polysaccharide sulphuric esters. The interrupted line is the slope previously obtained for the dextran sulphate series, for comparison.

The Nature of the Dye-substrate Interaction.

The observations of MacIntosh (1941), Jaques, Bruce-Mitford and Ricker (1949) and of Weissman *et al.* (1952) that slight alterations of ionic composition and concentration, pH, temperature and the dielectric constant affect the production of metachromasia, were confirmed during the present investigation. The effect of very high concentrations of the acid polysaccharide itself in apparently reversing metachromasia is illustrated in Table III.

TABLE III.—*Effect of Excess Dextran Sulphate on the Metachromatic Reaction.*

Dextran sulphate (mg./ml.).	200	100	50	25	12·5	6·25	3·1	1·6	0·8	0·0
Colour	Blue	Blue	Bluish purple	Bluish purple	Purple	Reddish purple	Reddish purple	Purple	Purple	Blue

1 ml. of 0·005 per cent toluidine blue at pH 2·04 added to doubling dilutions of dextran sulphate in 1 ml. vols. of 0·2 per cent NaCl.

It has been suggested that these factors operate by interfering with weak intermolecular forces causing aggregation of the dye molecules. Michaelis (1947) noted that toluidine blue departed from the Beer-Lambert Law in showing alterations of its absorption curve with concentration, and suggested that these alterations were due to progressive polymerization of the dye molecules. He observed that, with increasing concentration, the dye showed maximal absorption

first at 630 mμ (α band), then at 590 mμ (β band) and then successively betw en 540 mμ and 520 mμ (γ band) and suggested that the α band represented the monomeric form of the dye, the β band the dimeric form, and the wide γ band successively higher dye polymers. The resemblance between these changes in the absorption curve with simple increase of concentration and those occurring on the addition of anionic polyelectrolytes led Michaelis to propound the following hypothesis concerning the mechanism of metachromasia: that interaction occurs between the acidic groups of the substrate and the basic primary amine groups of the dye with the formation of ordinary salt linkages. This in itself does not necessarily result in metachromasia (μ absorption bands). But when the structure of the dye and of the substrate allow the bound molecules to come into close proximity, loose association occurs by van der Waal's forces between adjacent dye molecules (perhaps by way of interposed water molecules) thus bringing about virtual polymerization of the dye.

If in fact metachromasia were dependent, as suggested by Michaelis, on polymerization of the dye by its aggregation at selected sites upon the substrate, it would be expected that this process would be more marked with increase of polymerization of the substrate. From the present results it seems clear that, over a fairly wide range, the degree of polymerization of the polysaccharides examined does not influence the intensity of metachromasia.

The data from the present experiments allowed calculation of the nature of the interaction on a molecular basis. It was assumed that interaction occurred between the sulphate radicles of the dextran sulphates and the primary amine groups of the dye. The sulphur contents of a random selection of dextran sulphates of varying molecular characteristics, and the nitrogen content of the dye sample used, were estimated. It was thus possible to compare the actual weight of toluidine blue found experimentally to be bound by a given weight of each of the various dextran sulphates with the theoretical weight of dye which should be coupled by this weight of dextran sulphate. This calculation was performed assuming that one atom of sulphur (in each sulphate group) reacted with one nitrogen atom (per amine group) and repeated assuming $2S \equiv 3N$, $S \equiv 3N$ etc. (Table IV). It was found that good agreement occurred between the calculated and experimentally determined figures only when the reacting groups were assumed to contain one sulphur atom and three nitrogen atoms

TABLE IV.—*Comparison of Calculated and Experimentally Found Weights of Toluidine Blue Bound by 80 μg. of Various Dextran Sulphates.*

Dextran sulphates serial numbers.	Degree of polymerization (glucose units).	Sulphur content (per cent).	Toluidine blue bound (μg.). Predicted weight, assuming S ≡ N.	2S ≡ 3N.	S ≡ 3N.	Experimentally found.
AO	20	9·2	38·6	57·9	115·8	108·5
BB	20	11·8	49·5	74·3	148·5	145·0
E/1	35	13·8	55·6	83·4	173·0	174·0
BD	20	14·2	59·5	89·3	178·0	177·5
D 3	350	15·4	64·5	96·8	193·5	187·0
BE	20	15·9	66·5	99·8	199·5	198·7
I/4	20	17·2	72·5	108·8	217·5	203·7

Calculated values assume interaction in various proportions between sulphur atoms (in sulphate groups of polysaccharide) and nitrogen atoms (in amine groups of dye).

respectively (Table IV and Fig. 7). Since the molecule of toluidine blue contains three nitrogen atoms (Fig. 8), this was interpreted as implying coupling of a single molecule of the dye by each acidic group of the substrate.

This is in agreement with the later views of Michaelis (1950) who conceded that monomolecular distribution of the dye over the negatively charged sites of the colloid was more likely than the formation of polymolecular dye micelles. Levine and Schubert (1952*a*, *b*) have criticized the earlier hypothesis of Michaelis on data derived from equilibrium dialysis of thiazine dyes and various anionic polyelectrolytes producing metachromasia and have shown (Schubert and Levine, 1953), by a conductiometric method, that a correlation can be demonstrated between dye-binding and the intensity of metachromasia.

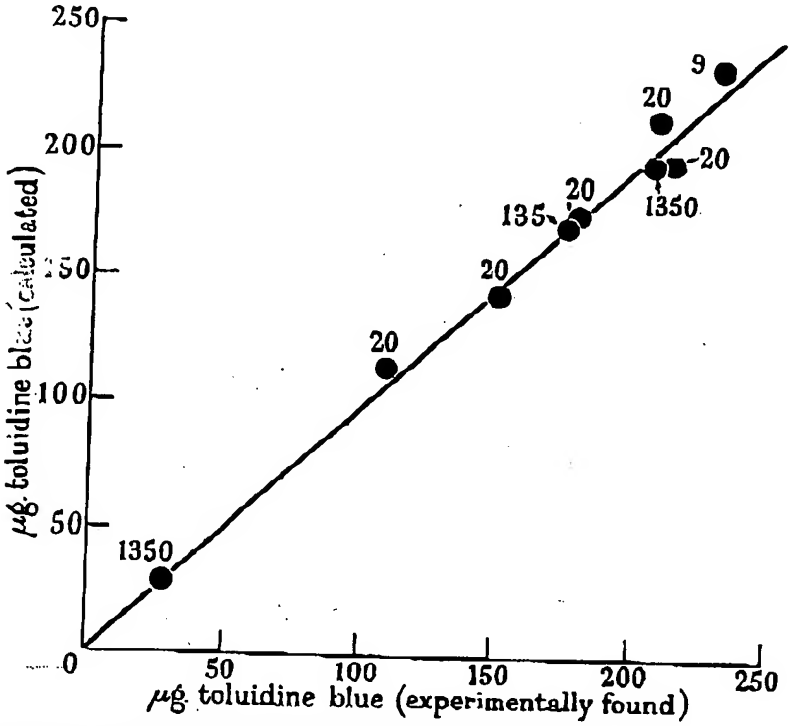

FIG. 7.—Comparison of calculated and experimentally found weight of toluidine blue (in μg.) combining with 80 μg. of various dextran sulphates assuming interaction occurs between one sulphur atom (per sulphate group) and three nitrogen atoms (per molecule of toluidine blue). The continuous line is the theoretical correlation. The plotted points indicate the values found.

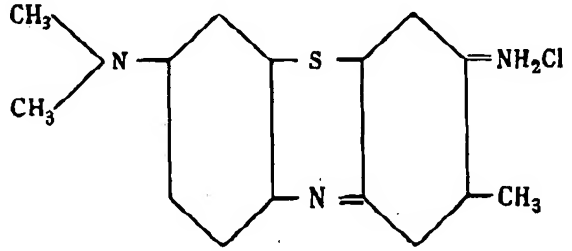

FIG. 8.—Structural formula of toluidine blue-O (from Conn, 1946).

DISCUSSION

Lison (1935*a*, *b*) suggested that the metachromatic reaction consisted essentially in the formation of the tautomeric imino-base of the dye accompanied by increased molecular aggregation and was characteristically brought about only by organic macromolecular substances containing ester sulphate radicles. The present findings confirm those quoted earlier in showing that other acidic radicles may also effect metachromasia and suggest that the intensity of the reaction is related to the extent of dissociation of these radicles. It was shown in Exp. 4 that the intensity of metachromasia produced by polycarboxylates was feeble relative to that produced by the polysulphates, at pH 2. At this level the carboxylate radicle is only slightly dissociated whereas the sulphate radicle is highly dissociated. Increase of pH to 7·3, bringing about further dissociation of the carboxylate group, resulted in increase of metachromasia, but even at this level the degree of dissociation of the carboxylate radicle is considerably less than that of the sulphate group, accounting for the difference observed.

From these experiments it would appear that pH and ionic strength affect the formation of the salt linkage between dye and substrate. Inorganic sulphates and even certain organic sulphonates such as polyanethol sulphonate ("Liquoid"), though they undoubtedly bind toluidine blue, do not produce any colour change (MacIntosh, 1941). It seems, therefore, that simple combination with the dye is not enough to produce metachromasia. The remaining factors known to influence this colour change (alteration of temperature, electrolyte or colloid concentration or of the dielectric constant) are those which are known to affect the solubility and state of aggregation of hydrophilic colloids (Glasstone, 1947).

With simple inorganic salts of high solubility, although dye-binding occurs, no colour change is observed. But with anionic polyelectrolytes, once a certain threshold of molecular size is exceeded (in glucose polymers, when the compound consists of 4 or more glucose-units), the dye-substrate complex acquires the properties of a hydrophilic colloid and aggregation of the dyed molecules produces a colour change similar to that seen with simple increase in concentration of the dye itself.

On this basis, the reversible inhibition of metachromasia which occurs on altering certain environmental factors of the reaction might be explained as follows: thermal agitation of the molecules (increase of temperature), or displacement of the water of hydration of the molecules and alteration of the conductance of the solution (alteration of the dielectric constant) by the addition of alcohol or increase of electrolyte concentration, would inhibit metachromasia not only by influencing the dissociation of the primary reacting groups but also by interference with the weak intermolecular forces causing association of the dyed molecules. On the other hand, the inhibition of metachromasia by considerable excess of substrate (Table II) would be due to competition between molecules of the substrate for dye molecules. As a result, the relatively few substrate molecules binding dye would be greatly outweighed by undyed molecules of substrate. Aggregation of substrate molecules would thus not be accompanied by colour change.

In histological use, metachromasia occurs in heterogeneous systems involving many complex variables which have not been considered in these experiments. In particular, no account has been taken of the modifying influence of protein

conjugated with mucopolysaccharide, though it may be assumed that such protein-polysaccharide association is usually present in the tissues. It has been shown by French and Benditt (1953) that dye-protein competition may occur when mucopolysaccharides are stained with basic dyes, so that, in the presence of a basic protein, the acidic character of the polysaccharide and hence the intensity of its metachromatic reaction may be partly or completely masked. In these circumstances the optimal pH of dye-binding by the mucopolysaccharide may be determined by the associated protein and reflect the competition between dye and protein for the acidic groups of the polysaccharide, rather than the dissociation characteristics of the acidic groups of the polysaccharides themselves. Similar conclusions were reached by Hamerman and Schubert (1953) who studied the metachromasia of hyaluronate and chondroitin sulphate in the presence and absence of added protein.

The findings of these authors, in conjunction with those of the present investigation suggest that : (*a*) If under experimental conditions, tissue components show *decreased* metachromasia, this may mean

(i) Decrease in concentration of the acidic polysaccharide ; or
(ii) Loss of acidic groups or binding of acidic groups by protein ; or
(iii) Disruption of the molecular structure of the acidic polysaccharide or, at least, degradation to diffusible fractions ;

but *not* partial depolymerization of the polysaccharide.

(*b*) Conversely, *increased* metachromasia occurring under similar conditions may mean :

(i) Increased concentration of the acidic polysaccharide ; or
(ii) Increase in the number of acidic groups or their unblocking by dissociation of a protein-mucopolysaccharide complex ;

but *not* increase of polymerization of the polysaccharide.

SUMMARY

The determinants of the intensity of metachromasia have been investigated by quantitative measurements of the interaction between toluidine blue and different series of acidic polysaccharides. The latter compounds varied (*a*) in the extent of their polymerization, (*b*) in the number and nature of the attached acidic radicles, and (*c*) in the nature of their component saccharide units.

It was found that the intensity of metachromasia is directly related to the number and the dissociation characteristics of the attached acidic radicles but not to the nature, disposition or number of the constituent units for glucose polymers containing more than four saccharide units.

It is suggested that metachromasia is not dependent on the extent of polymerization of the dye molecules on the surface of the substrate nor on the extent of polymerization of the acidic polysaccharide itself but on the solubility characteristics of the dye-substrate complex.

The significance of these results in relation to the interpretation of alterations of metachromasia in histological use is discussed.

An abridged account of this investigation formed part of a communication given by one of us (K. W. W.) before the Royal Society of Medicine on October 20, 1953. We are obliged to the Society for permission to publish the present

communication in this form. We are grateful to Professor J. R. Squire and Dr. G. A. Gilbert for their suggestions, comments and criticisms of the manuscript, and to Mr. J. Hazlewood for the preparation of the illustrations.

REFERENCES.

BALL, J., AND JACKSON, D. S.—(1953) *Stain Tech.*, **28**, 33.
CAMPANI, M., AND REGGIANI, O.—(1950) *J. Path. Bact.*, **62**, 563.
CONN, H. J.—(1946) 'Biological Stains,' 5th ed. Geneva N.Y. (Biotech. Publications), p. 97.
CURRAN, R. C.—(1953) *J. Path. Bact.*, **66**, 271.
FRENCH, J. E., AND BENDITT, E. P.—(1953) *J. Histochem. Cytochem.*, **1**, 321.
GERSH, I.—(1951) 'Connective Tissues,' Ragan, C. A., Jr., Editor, Trans. Second Conf. New York (Josiah Macy Jr. Foundation), p. 15.
GLASSTONE, S.—(1947) 'Textbook of Physical Chemistry,' 2nd ed. London (Macmillan).
HAMERMAN, D. J., AND SCHUBERT, M.—(1953) *J. gen. Physiol.*, **37**, 291.
JAQUES, L. B., BRUCE-MITFORD, MARGARET, and RICKER, ANN G.—(1949) *Rev. Canad. Biol.*, **6**, 740.
KELLEY, E. G., AND MILLER, E. G., Jr.—(1935) *J. biol. Chem.*, **110**, 113.
LENNOX, B., PEARSE, A. G. E., AND RICHARDS, H. G. H.—(1952) *J. Path. Bact.*, **64**, 865.
LEVINE, ANNE, AND SCHUBERT, M.—(1952*a*) *J. Amer. chem. Soc.*, **74**, 91.—(1952*b*) *Ibid.*, **74**, 5702.
LISON, L.—(1935*a*) *Bull. Histol. Tech. micr.*, **12**, 279.—(1935*b*) *Arch. Biol., Paris*, **46**, 599.
LOEWI, G.—(1953) *J. Path. Bact.*, **65**, 381.
MACINTOSH, F. C.—(1941) *Biochem. J.*, **35**, 776.
MERRILL, R. C., SPENCER, R. W., AND GETTY, R.—(1948) *J. Amer. chem. Soc.*, **70**, 2460.
MEYER, K.—(1947) *Physiol. Rev.*, **27**, 335.
MICHAELIS, L.—(1930) *J. biol. Chem.*, **87**, 33.—(1947) *Cold Spr. Harb. Symp. quant. Biol.*, **12**, 131.—(1950) *J. phys. Chem.*, **54**, 1.
PENNEY, J. R., AND BALFOUR, BRIGID M.—(1949) *J. Path. Bact.*, **61**, 171.
RICKETTS, C. R.—(1952*a*) *Biochem. J.*, **51**, 129.—(1952*b*) *Chem. and Ind. (Rev.)*, p. 982.
SCHUBERT, M., AND LEVINE, ANNE—(1953) *J. Amer. chem. Soc.*, **75**, 5842.
SYLVÉN, B., AND MALMGREN, H.—(1952) *Laboratory Investigation*, **1**, 413.
WALTON, K. W.—(1952) *Brit. J. Pharmacol.*, **7**, 370.—(1953) *Ibid.*, **8**, 340.
WEISSMAN, N., CARNES, W. M., RUBIN, P. S., AND FISHER, JEAN—(1952) *J. Amer. chem. Soc.*, **74**, 1423.
WIAME, J. M.—(1947) *Ibid.*, **69**, 3146.
WISLOCKI, G. B., BUNTING, H., AND DEMPSEY, E. W.—(1947) *Amer. J. Anat.*, **81**, 1.